J. Clin. Chem. Clin. Biochem.
Vol. 18, 1980, pp. 413–421

Single-Isotope Enzymatic Derivative Method for Measuring Catecholamines in Human Plasma[1])

By *J. Bosak, E. Knoll, D. Ratge* and *H. Wisser*

Department of Clinical Chemistry, Robert-Bosch-Krankenhaus, Stuttgart

(Received October 1, 1979/January 21, 1980)

Summary: The radioenzymatic determination of plasma catecholamines with a modification of the method of *da Prada & Zürcher* ((1976), Life Sci *19*, 1161–1174) is described. The several reaction steps were optimized with respect to the quantities of substrate and enzyme, and reaction time. There were particular methodological difficulties concerning the blanks, which were determined by using sodium metaperiodate-oxidized plasma. The reliability criteria of the method were determined. Coefficients of variation between 3.4 and 8.6% were found for the intra-assay variability of 10 pg of norepinephrine and 3 pg of epinephrine or dopamine, resp. The recoveries of the three catecholamines ranged from 88–93%. The detection limits were calculated from the standard deviation of the blanks and amounted to 12 ng/l (norepinephrine), 6 ng/l (dopamine) and 3 ng/l (epinephrine). The method was used for the analysis of plasma samples from patients. In a further investigation we examined the stability of plasma catecholamines stored at different temperatures. It was found that samples can be stored for 1–2 hours at room temperature and for several weeks at – 27 °C without losses in catecholamine content.

Enzymatische Einisotopenderivat-Methode zur Bestimmung der Catecholamine im Plasma

Zusammenfassung: Die radioenzymatische Bestimmung der Catecholamine im Plasma mit einer Modifikation der Methode von *da Prada & Zürcher* ((1976), Life Sci *19*, 1161–1174) wird beschrieben. Die einzelnen Reaktionsschritte wurden bezüglich der Substrat- und Enzymmenge sowie der Reaktionsdauer optimiert. Besondere methodische Schwierigkeiten gab es bei den Leerwerten, für die mit Natriummetaperjodat oxidiertes Plasma eingesetzt wurde. Die Zuverlässigkeitskriterien der Methode wurden ermittelt. Die Streuung in der Serie für 10 pg Noradrenalin und jeweils 3 pg Adrenalin und Dopamin lag zwischen 3,4 und 8,6%. Die Wiederfindung bei Aufstockexperimenten betrug 88–93%. Aus der Streuung der Leerwerte wurden die folgenden Nachweisgrenzen berechnet: Noradrenalin 12 ng/l, Dopamin 6 ng/l und Adrenalin 3 ng/l. Plasmaproben von Patienten wurden mit dem Verfahren analysiert. Außerdem wurde die Stabilität der Catecholamine im Plasma bei verschiedenen Temperaturen untersucht. Nach den Ergebnissen dieser Versuchsreihe können Plasmaproben 1–2 Stunden bei Raumtemperatur und mehrere Wochen bei – 27 °C ohne Verluste an Catecholaminen aufbewahrt werden.

Introduction

The development of a double-isotope derivative method by *Engelman* and coworkers (1, 2) was an important methodological advance in the field of catecholamine analysis. With this method epinephrine and norepinephrine in plasma could be determined specifically; the precision, however, was unsatisfactory, and the epinephrine concentrations in human plasma were near the detection limit of the assay.

The method is based on the enzymatic conversion of the catecholamines with S-adenosyl-*L*-methionine-(methyl[^{14}C]) and catechol-O-methyltransferase to their 3-O-methylated derivatives. Losses in the course of the assay are corrected for by addition of tritiated epinephrine and norepinephrine to the plasma. *Passon & Peuler* (3) introduced an important modification of this method by using S-adenosyl-*L*-methionine-(methyl[^{3}H]) which has a considerably higher specific activity than the S-adenosyl-*L*-methionine(methyl-[^{14}C]). This resulted in a 10-fold improvement of the assay sensitivity so that the sample volume could be reduced from 10 ml to less than 1 ml plasma. The losses were corrected for by use of internal standards. Dopamine in plasma was first determined radioenzymatically by *Coyle & Henry* (4). In the last few years a series of reports has appeared dealing with the radio-

[1]) This investigation was supported by the Robert Bosch Stiftung, Stuttgart (FRG).

0340-076X/80/0018-0413$2.00

enzymatic determination of the catecholamines (5–13). The present report describes a modification of the *da Prada-Zürcher*-method (6); the conditions of the enzymatic methylation were investigated, the reliability criteria were determined, and applicability to the analysis of human plasma was examined.

Materials and Methods

Principle of the method

By modifying the procedure of *da Prada & Zürcher* (6), a radioenzymatic method was developed for the simultaneous determination of epinephrine, norepinephrine and dopamine. It is based on the enzymatic 3-O-methylation of the three catecholamines by catechol-O-methyltransferase (EC 2.1.1.6) in the presence of S-adenosyl-methionine(methyl[^{3}H]) of a very high specific activity (2.04–3.15 TBq/mmol = 55–85 Ci/mmol) to [^{3}H]metanephrine, [^{3}H]normetanephrine and [^{3}H]methoxytyramine respectively. After addition of sodium tetraphenylborate the tritiated 3-methoxy-derivatives are extracted into diethyl ether as tetraphenylborate-complexes. They are then nearly quantitatively re-extracted into hydrochloric acid. The reaction products are separated by thin-layer chromatography. The periodate oxidation of [^{3}H]metanephrine and [^{3}H]normetanephrine to vanillin results in a further reduction of the blanks. [^{3}H]methoxytyramine lacking the β-hydroxyl group, does not oxidize under these conditions. It is therefore converted into the diacetyl derivative by reaction with acetic anhydride. The resulting compounds are extracted into toluene and the tritium activity is counted in a liquid scintillation spectrometer. Figure 1 shows the principle of the method.

Description of the procedure

Preparation of catechol-O-methyltransferase (EC 2.1.1.6) from rat liver according to Axelrod & Tomchick (14)

8 rats were decapitated, the livers (60–70 g) immediately removed and transferred into the 4-fold volume (w/v) of icecold isotonic KCl-solution. All further purification procedures are carried out at 0–5 °C. The liver is minced and homogenized with the KCl-solution, followed by 30 min centrifugation at 80000 *g*.

The supernatant is filtered, adjusted to pH 5 with 1 mol/l acetic acid and stirred for 20 min. The suspension is centrifuged at 10000 *g* for 15 min. The precipitate is discarded, the supernatant ist fractionated with ammonium sulfate. Degree of saturation 0–0.3: 0.174 g ammonium sulfate are added per ml supernatant; the mixture is stirred for 20 min and centrifuged at 10000 *g* for 30 min. The precipitate is discarded. Degree of saturation 0.3–0.5:0.177 g ammonium sulfate are added per ml supernatant; the mixture is again stirred for 20 min followed by 30 min centrifugation at 18000 *g*. The supernatant is discarded and the precipitate is dissolved in 30–40 ml of sodium phosphate buffer (1 mmol/l) pH 7. The solution is dialyzed for 12 h against 1:5 l of the phosphate buffer containing 0.1 mmol/l dithiothreitol. The precipitate formed during dialysis is centrifuged for 15 min at 10000 *g*. The supernatant (40 ml) is dispensed in 1 ml-portions in disposable containers (Eppendorf tube) and stored at – 27 °C until use. The protein concentration of the solution is 14.9 mg/ml (biuret-method).

Reagents and solutions

Ethyleneglycol-bis (β-aminoethylether) N,N'-tetraacetic acid (EGTA), *DL*-normetanephrine hydrochloride, *DL*-metanephrine hydrochloride, 3-methoxytyramine hydrochloride, *DL*-dithiothreitol: Sigma Chemie, Taufkirchen, FRG; *L*-norepinephrine hydrogentartrate: Fluka, Buchs/Switzerland; *L*-epinephrine base, glutathione red.: Merck, Darmstadt, FRG;

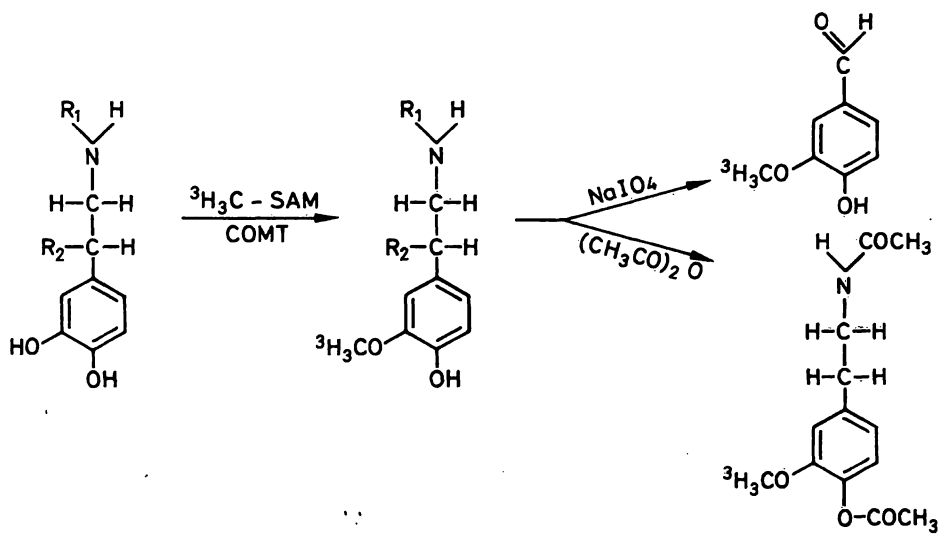


Fig. 1. Schematic representation of the major steps of the radioenzymatic catecholamine assay.
SAM = S-adenosyl-*L*-methionine;
COMT = catechol-O-methyltransferase.

Norepinephrine: R_1 = H, R_2 = OH
Dopamine: R_1, R_2 = H
Epinephrine: R_1 = CH_3, R_2 = OH

dopamine hydrochloride: Serva, Heidelberg, FRG; 2-(4'-tert.-butylphenyl)-5-(4''-biphenyl)-1,2,3-oxadiazole (butyl-PBD) (4 g/l toluene), Riasolve I: Zinsser, Frankfurt/M., FRG; [7-^{3}H (N)]*DL*-normetanephrine (specific activity 0.48 TBq/mmol = 13 Ci/mmol), [7-^{3}H (N)]*DL*-metanephrine (specific activity 0.34 TBq/mmol = 9.3 Ci/mmol), [5-^{3}H (N)]3-methoxytyramine (specific activity 0.41 TBq/mmol = 11 Ci/mmol), S-adenosyl-*L*-methionine (methyl[^{3}H]) (specific activity 2.04–3.15 TBq/mmol = 55–85 Ci/mmol): NEN, Dreieich, FRG. All other reagents used were of analytical grade and obtained from Merck (Darmstadt) or Riedel de Haen (Hannover, FRG). 2.5 g/l EGTA and 2.5 g/l $MgCl_2 \cdot 6\,H_2O$ in 0.6 mol/l perchloric acid (solution was prepared immediately before use). All other solutions were renewed every 2–3 weeks. Kieselgel 60 F_{254} plates (250 µm thickness, 5 × 10 cm, Merck) were used for thin-layer chromatography.

Solvent system: Chloroform/methanol/ethylamine 70% (80 ml/15 ml/10 ml).

Standard solutions

Stock solutions: 5 mg catecholamine (free base) in 20 ml 0.01 mol/l HCl. These solutions are stored at 4 °C. The working solutions are prepared by dilution of the stock solutions with 0.01 mol/l HCl; e.g. a 1:125 000 dilution of the stock solution (12.5 µg/50 µl) results in a working solution with a catecholamine-concentration of 100 pg/50 µl.

Sample collection

6 mg glutathione (1.5 mg/ml) were added in 4 ml Monovettes® (Sarstedt, Nümbrecht-Rommelsdorf, FRG) and stored at 4 °C. 100 µl Liquemin® (500 USP-U sodium heparin, Hoffmann La Roche, Switzerland) are added shortly before blood sampling. Blood samples were obtained from cubital veins of supine patients. The Monovettes® were placed on ice immediately after collection and centrifuged within 10 min at 4 °C for 15 min. Plasma was separated from the erythrocytes as quickly as possible and deproteinized with an equal volume of 0.6 mol/l perchloric acid containing EGTA (2.5 g/l) and $MgCl_2 \times 6\,H_2O$ (2.5 g/l). After centrifugation for 15 min at 4 °C the supernatant was directly used for the catecholamine determination or stored at – 27 °C until analysis. The essential steps of the assay are summarized in figure 2.

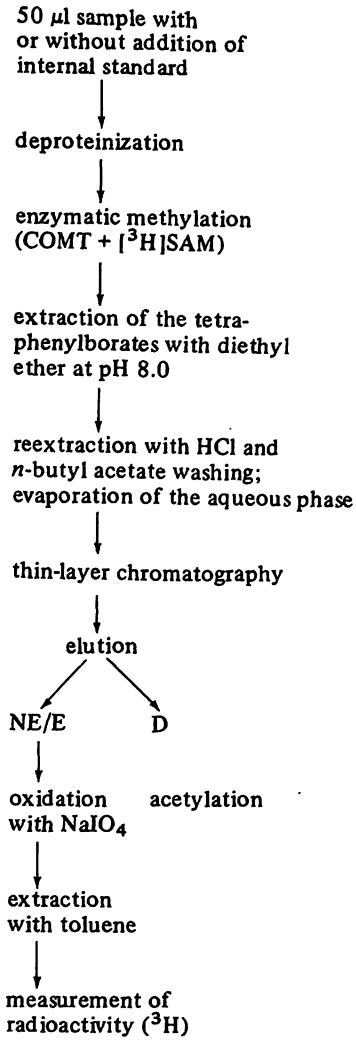


Fig. 2. Schematic representation of the working procedure of the radioenzymatic catecholamine assay.

100 µl of the supernatant (corresponding to 50 µl plasma) or 100 µl 0.3 mol/l perchloric acid (assay of pure aqueous solutions) are pipetted in conical glass tubes, prechilled in an ice-water bath. This is followed by 50 µl standard working solution (samples with internal standard) or 50 µl 0.01 mol/l HCl (samples without internal standard and blanks). The reaction is started by the addition of the following mixture: 0.1 mg dithiothreitol in 50 µl 2 mol/l tris-HCl buffer, 20 µl 25 mmol/l $MgCl_2 \times 6\,H_2O$, (92,5 kBq = 2.5 µCi)S-adenosyl-*L*-methionine (methyl[^{3}H]) (5 µl; 30–45 pmol) and 25 µl of the catechol-O-methyl-transferase preparation. (This solution is prepared in an ice-cold bath just before use and is shaken carefully, avoiding foaming). Dithiothreitol should be weighed out with a plastic or glass spatula because the thiol-groups react with heavy metals. The tubes are closed, then mixed thoroughly (Vortex) and incubated for 60 min at 37 °C in a shaking water bath. The reaction mixture has a pH of 8.15–8.20. After incubation the tubes are placed back into the ice-bath and the reaction is stopped by addition of 200 µl of a mixture of 1 mol/l borate buffer pH 8 and of a carrier solution (3/1 v/v). The carrier solution contains 0.5 g/l 0.01 mol/l HCl of the cold methylated derivatives of the catecholamines (metanephrine, normetanephrine and 3-methoxytyramine). The mixture of the borate buffer and the carrier solution is prepared freshly before use. After addition of 50 µl sodium tetraphenyl-borate solution (15 g/l), the solution is mixed gently with a Vortex and then extracted with 10 ml cold water-saturated diethyl ether by rotating the tubes for 10 min. The phases are separated by low-speed centrifugation (3 min). Now the tubes are quick-frozen, five at a time, in an acetone-dry ice bath for 30–60 s. The aqueous phase must be completely frozen. It is important that the aqueous layer is completely submerged in the acetone-dry ice bath and that the organic layer is above the level of the bath. If the tubes are left in the bath too long, the water dissolved in the diethyl ether may also freeze (cloudiness). This results in a large deviation of the analyses. The diethyl ether is decanted into a set of new tubes containing 0.5 ml 0.1 mmol/l HCl. These tubes are again rotated for 10 min, centrifuged and quick-frozen. The organic layer is discarded and the frozen aqueous phase is washed with 2 ml diethyl ether. The hydrochloric acid phase is then thawed, 5 ml water saturated *n*-butyl-acetate are added and mixed vigorously with a Vortex mixer for 30 s. The phases are separated by low-speed centrifugation and the organic phase is aspirated and discarded. The *n*-butyl acetate washing is repeated. The aqueous phases are evaporated to dryness, 6 samples at a time, under high vacuum in a rotary evaporator in a water bath at 20 °C.

The residue is taken up with 100 µl of a mixture of methanol and 0.01 mol/l HCl (5:1 v/v) containing 1 µl β-mercaptoethanol per ml as an antioxidant. This solution is spotted on a Kieselgel 60 F_{254} plate in a band approximately 0.5 × 3 cm under a stream of cold air. The chromatogram is developed for 25–30 min in a mixture of chloroform/methanol/ethylamine 70% (16 ml/3 ml/2 ml). The plates are dried thoroughly under a stream of cold air and the bands are localized under UV light (254 nm). The silica gel of the three spots corresponding to metanephrine, normetanephrine and 3-methoxytyramine are scraped off into Eppendorf tubes. 1.3 ml of 2 mol/l NH_3 solution is added to the tubes corresponding to metanephrine and normetanephrine, followed by shaking for 10 min

to elute the methylated catecholamines. After centrifuging for 2 min with the Eppendorf-centrifuge (15000 *g*) 1 ml of the supernatant is transferred to new glass-stoppered tubes. The precipitate is resuspended in 0.5 ml 2 mol/l NH_3 solution and after shaking for 5 min and centrifuging for 2 min 0.6 ml of the supernatant are combined with the first NH_3 eluate. 3-Methoxytyramine is eluted with 0.01 mol/l HCl in the same way as described for metanephrine and normetanephrine.

100 µl of a 40 g/l $NaIO_4$-solution are added to the tubes with the metanephrine and normetanephrine eluates. After mixing with a Vortex mixer the tubes are shaken gently for 10 min at room temperature. The reaction is stopped in an ice-cold bath after 10 min by the addition of 400 µl of 10 mol/l acetic acid (pH 6). The tubes are again shaken for a short time and then 10 ml toluene are added to each tube. The vanillin, formed by metaperiodate oxidation of metanephrine and normetanephrine is extracted into toluene by shaking the tubes for 10 min on a mechanical shaker. The phases are separated by low-speed centrifugation for 3 min and quick-freezing of the lower aqueous phase. The toluene phase is decanted in polyethylene counting vials, being prefilled with 5 ml of butyl-PBD-toluene scintillation fluid. The vials are capped, shaken vigorously and counted in a liquid scintillation spectrometer (Mark II, Searle Anal.) for 10 min. The counting efficiency is 57–59%.

The 3-methoxytyramine eluate is acetylated at room temperature by Vortex-mixing for 30 s with 0.16 g sodium bicarbonate and 200 µl of acetic anhydride. After exactly 5 min the excess sodium bicarbonate is neutralized with glacial acetic acid. The solutions are allowed to stand for 30 min to ensure the hydrolysis of the excess acetic anhydride. Subsequent treatment of the samples is as described for metanephrine and normetanephrine after the periodate oxidation.

Calculation

The quantitative evaluation of the samples is based upon the radioactivity of the internal standard.

$$\frac{cpm_{sample} - cpm_{blank}}{cpm_{(sample + standard)} - cpm_{sample}} \times \frac{pg_{standard}}{ml_{sample}} = \frac{pg_{catecholamine}}{ml_{sample}} =$$

ng/l catecholamine in the sample

Results and Discussion

Enzymatic methylation

First, the optimal conditions of the different steps of the enzymatic methylation were determined. We investigated the dependence of the yield of normetanephrine on the concentrations of S-adenosyl-methionine, norepinephrine, and Ca^{2+}, on the quantity of catechol-O-methyl-transferase and the reaction time. Pure aqueous solutions of norepinephrine were used for these investigations. These measurements were performed fluorimetrically with cold S-adenosyl-*L*-methionine according to *Axelrod & Tomchick* 14, 15). As a result of these investigations 0.5 mg catechol-O-methyltransferase (referring to the protein content), a reaction time of 60 min and a 10 fold excess of S-adenosyl-*L*-methionine (in relation to the substrate norepinephrine) were necessary for an optimal methylating reaction. 60 nmol of norepinephrine were used for these measurements. The efficiency of the enzymatic

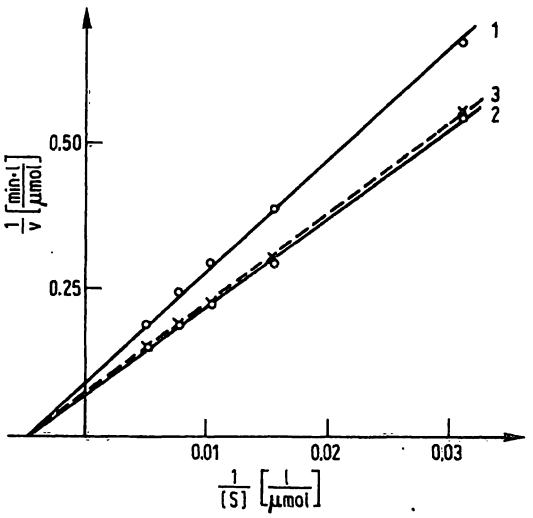


Fig. 3. Inhibition of the enzyme reaction of norepinephrine after addition of Ca^{2+} (curve 1) and removal of the inhibition by the addition of EGTA (curve 2). Curve 3 demonstrates the enzymatic reaction of norepinephrine in the presence of EGTA but without Ca^{2+}.

methylation was 80%. With the radioenzymatic examination of this reaction we got lower recoveries for norepinephrine (70%) and dopamine (55%) and a similar recovery for epinephrine (85%). Figure 3 demonstrates the inhibition on the methylation by Ca^{2+} and its removal by addition of the Ca^{2+} chelating agent EGTA.

Curve 1 shows the inhibition by Ca^{2+} of the enzyme reaction (0.26 µmol Ca^{2+}). Using 50 µl of plasma as starting volume, 0.26 µmol Ca^{2+} are equivalent to the upper limit of the normal range of calcium in plasma. Curve 2 shows the prevention of the inhibitory effect of Ca^{2+} by the addition of 0.52 µmol of EGTA and curve 3 demonstrates the course of reaction in the presence of EGTA but without Ca^{2+}. These investigations prove that the inhibition of catechol-O-methyltransferase by Ca^{2+} is completely removed by addition of EGTA.

Purification of the 3-methoxy-derivatives

Oxidation of the metanephrines with sodium metaperiodate or acetylation of 3-methoxytyramine with acetic anhydride are important steps after the thin-layer chromatographic separation of the 3-methoxy derivatives. The additional purificaton steps result in a considerable reduction of the blanks.

Da Prada & Zürcher (6) oxidized the metanephrines in 2 mol/l NH_3 (about 1.8 ml eluate) with 0.25 ml of 30 g/l sodium metaperiodate solution for 10 min at 37 °C. These authors reported recoveries of 86 and 73% for metanephrine and normetanephrine resp. We found recoveries of 73 ± 2% for both compounds. Our attempts to enhance the recovery by variation of the reaction conditions (volume of oxidant and extraction agent or reaction temperature) resulted in no significant improvement.

In contrast to *da Prada & Zürcher*, who directly measured the radioactivity of the 3-methoxytyramine eluate after thin-layer chromatography, and in accordance with some other authors (16, 17), we introduced the acetylation of 3-methoxytyramine as an additional purification step. This results in an improvement of the counting efficiency (from 22% to 57%) and in a reduction of the blanks.

Blanks

As mentioned previously we intended to increase the sensitivity of the method by use of S-adenosyl-*L*-methionine(methyl[^{3}H]) with a very high specific activity (2.04–3.15 TBq/mmol = 55–85 Ci/mmol). However, this introduced problems regarding the blanks. They showed great inter-assay variability, though we used the same solutions and S-adenosyl-*L*-methionine-(methyl[^{3}H]) of the same lot. Therefore we ran 3 different blanks in one assay:

1. Plasma oxidized with sodium metaperiodate (2 mg/ml) for 10 min at 37 °C and subsequently deproteinized with perchloric acid (0.6 mol/l).
2. The complete incubation mixture without the catechol-O-methyl-transferase is added to the deproteinized plasma (the enzyme is replaced by an albumin solution with the same protein concentration).
3. Plasma, which had been allowed to stand for 14 days at room temperature.

The efficiency of the various isolation steps was examined by measuring the radioactivity of aliquots after the extractions. The results of these measurements are summarized in table 1; they are expressed in dpm (Bq) to eliminate variable quench effects.

As can be seen from this table, the greatest differences occur at the first separation steps, the extraction of the tetraphenylborates with diethyl ether. The high blank 3 is possibly caused by the slower decomposition of the catecholamines bound to plasma proteins.

An additional washing of the diethyl ether phase with 0.1 mol/l borate buffer, or a second washing of the HCl phase with butyl acetate, or the application of different developing solutions brought no further reduction of the blanks. Table 2 illustrates the various solvent systems which were tested, the R_f-values of the methylated catecholamines and the development times.

Due to the short time required for development and the good separation, solvent system 1 was used for subsequent investigations. From the 3 blanks tested, we chose blank 1 for our experiments.

Losses at various separation steps

The radioenzymatic determination of the catecholamines consists of a series of separation steps as seen in figure 2. The losses at the different steps were determined by measuring the recoveries of [^{3}H]metanephrine, [^{3}H]-normetanephrine and [^{3}H]3-methoxytyramine in percent of the initial radioactivity. The results are shown in table 3.

The results are the mean of 3 determinations. As can be seen from this table the greatest losses occur during thin-layer chromatography and subsequent elution of the methylated derivatives from the silica gel.

Tab. 1. Gradual reduction of 4 different blank values throughout various separation steps. Blanks 1, 2 and 3 refer to the 3 blanks described in the text. The initial radioactivity was 92.5 kBq = 2.5 μCi. The results are given in min^{-1} and s^{-1} (Bq)!

	1 min^{-1} (Bq)	2 min^{-1} (Bq)	3 min^{-1} (Bq)	4+) min^{-1} (Bq)
Diethyl ether extraction	52923 (882)	58520 (975)	290688 (4845)	183547 (3059)
HCl-extraction	13225 (220)	22359 (373)	44352 (739)	44397 (740)
1. *n*-butyl acetate washing	10312 (172)	19276 (321)	30150 (503)	32720 (545)
2. *n*-butyl acetate washing	9097 (152)	17657 (294)	24031 (401)	–
Elution from silica gel				
Normetanephrine	1488 (24.8)	2716 (45.3)	2351 (39.2)	364 (6.1)
Metanephrine	1142 (19.0)	1674 (27.9)	2267 (37.8)	205 (3.4)
3-Methoxytyramine	571 (9.5)	744 (12.4)	927 (15.5)	612 (10.2)
After oxidation or acetylation				
Normetanephrine	30 (0.50)	43 (0.72)	183 (3.05)	43 (0.72)
Metanephrine	28 (0.47)	27 (0.45)	216 (3.60)	45 (0.75)
3-Methoxytyramine	40 (0.67)	47 (0.78)	228 (3.80)	–

+) according to *da Prada & Zürcher*.

Tab. 2. R_f-values of the methylated catecholamine derivatives and development times of the 3 solvent systems tested:

Solvent system 1: Chloroform:methanol:ethylamine 70% (80 ml/15 ml/10 ml)

Solvent system 2: tert.-amylalcohol:benzene:methylamine 40% (60 ml/20 ml/30 ml)

Solvent system 3: sec.-amylalcohol:benzene:chloroform:methanol:ethylamine (60 ml/20 ml/25 ml/40 ml/7.5 ml)

Solvent System	R_f-Values Normetanephrine	Metanephrine	3-Methoxytyramine	Development time (min)
1	0.29	0.42	0.61	24
2	0.18	0.27	0.37	55
3	0.38	0.49	0.63	35

Tab. 3. Recoveries of [^{3}H]normetanephrine, [^{3}H]metanephrine and [^{3}H]methoxytyramine at various separation steps. The recoveries of the three compounds were determined individually.

Separation steps	Recovery (%)		
	[^{3}H]Normetanephrine	[^{3}H]Metanephrine	[^{3}H]Methoxytyramine
Diethyl ether extraction	75.1 ± 2.8	79.5 ± 1.6	82.4 ± 3.8
0.1 mol/l HCl-extraction	74.7 ± 1.7	78.0 ± 0.8	77.5 ± 4.2
n-butyl acetate washing	73.5 ± 2.1	76.7 ± 0.7	75.9 ± 1.7
Elution from silica gel	44.8 ± 0.9	48.5 ± 1.3	53.9 ± 3.5
Oxidation or acetylation and toluene extraction	33.6 ± 1.2	36.4 ± 1.9	40.4 ± 3.3

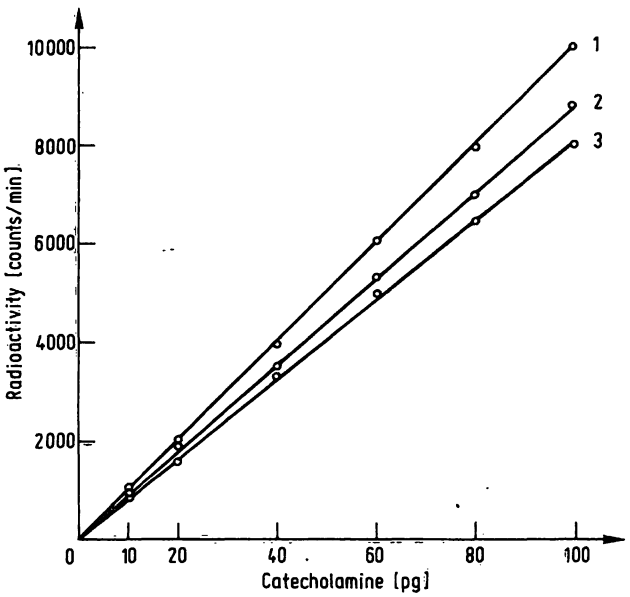


Fig. 4. Relationship between the measured radioactivity and the starting quantities of the catecholamines.
1. Epinephrine 2. Dopamine 3. Norepinephrine.

Reliability criteria of the method

Intra-assay variability

A human plasma pool was used for the determination of the intra-assay variability. 10 pg norepinephrine, 3 pg epinephrine and 3 pg dopamine were added per 50 µl of plasma. Seven samples were analyzed in one run. The results are depicted in table 4.

Variation coefficients between 3.4 and 8.6% were found for the intra-assay variability.

Accuracy

The linearity of the assay was examined with aqueous solutions of the catecholamines and plasma samples. Figure 4 shows the measured values of the plasma with different additions of known amounts of catecholamines.

Each value is the mean of 3 determinations. As can be seen from figure 4 the assay is linear in the range from 0–100 pg. Measurements on aqueous solutions of catecholamines also revealed a linear relationship between the counts/min and the concentration up to 150 pg. Recovery experiments with different additions of catecholamines to a plasma pool were also carried out. If the calculation was based on a standard curve measured some days before, the recoveries ranged from 103–130%. The greatest deviations occurred with dopamine. If the calculation was based on the radioactivity of the internal standards, we obtained recoveries between 88 and 93%.

Detection limit

The detection limit can be calculated from the threefold standard deviation of the blanks (18). We determined it by running 10 blanks in one series.

As shown in table 5 the detection limit for norepinephrine is 12 ng/l, for dopamine 6 ng/l and for epinephrine 3 ng/l. Other authors (12) defined the detection limit as a sample-to-blank ratio of 2. Table 6 shows a comparison between some radioenzymatic catecholamine-assays published recently, with special reference to the sensitivity.

Tab. 4. Intra-assay variability (n = 7).

	Blank	Sample				
	$\bar{x}$ (counts/min)	$\bar{x}$ (counts/min)	s (counts/min)	$\bar{x}$ (counts/min · pg)	s (counts/min · pg)	VK (%)
10 pg Norepinephrine	199	996	27	79.9	2.7	3.4
3 pg Epinephrine	142	454	18	104.0	6.0	5.8
3 pg Dopamine	343	611	23	89.3	7.9	8.6

Tab. 5. Detection limit of the assay.
(Calculation based upon the intra-assay variability of the blanks (18))

Compound	Blanks (counts/min) $\bar{x}$	±	s	3 s	$\bar{x}$ (counts/ min · pg)	Detection limit pg/50 µl	ng/l
Norepinephrine	266	±	16.7	50.1	77	0.61	12.2
Epinephrine	155	±	5.8	17.4	98	0.18	3.6
Dopamine	163	±	10.3	31.0	90	0.32	6.4

Tab. 6. Sensitivity of the radioenzymatic determination of catecholamines as reported by various authors.

	Engelman et al. (1970)	*Passon & Peuler* (1973)	*Da Prada & Zürcher* (1976)	*Peuler & Johnson* (1977)	*Saller & Zigmond* (1978)	*Bosak* et al. (1980)
counts/min · ng Norepinephrine	42	438	28973/29000	33000	61980/18200	79700
(counts/min blanks)	(11)	(10)	(18)	(26)	(525/52)	(17)
counts/min · ng Epinephrine	44	767	28502/35000	39000	61108/16152	104000
(counts/min blanks)	(11)	(10)	(18)	(33)	(694/56)	(16)
counts/min · ng Dopamine	–	–	21677/19000	33000	61416/19236	89300
(counts/min blanks)			(32)	(180)	(529/64)	(23)
Specific activity of S-adenosyl-*L*-methionine(methyl[^{3}H])						
(Ci/mmol)	0.048–0.055	4	7–10	9–11	80	60–80
(GBq/mmol)	1.78–2.04	148	259–370	333–407	2960	2220–2960
Detection limit (pg)	250	20/13	0.6/0.6/1.5	0.75/0.85/6.5	8.5/11.3/8.5 (2.85/3.47/3.33)	0.20/0.15/0.26
Sample volume (µl)	10000	500	50	50	10	50
Detection limit (ng/l)	25	40/26	12/12/30	15/17/110	850/1130/850 (285/347/333)	4/3/5

As can be seen from these values, the sensitivity of the assays increased with the availability of higher specific activity of S-adenosyl-*L*-methionine(methyl[^{3}H]). Moreover some authors report detection limits which are above the resting catecholamine concentrations in human plasma.

Application of the assay

Resting plasma catecholamine concentrations

Blood samples were taken from 4 volunteers, which had been recumbent for 30 min. The following catecholamine concentrations were determined: epinephrine 26–85 ng/l, norepinephrine 73–215 ng/l and dopamine 9–37 ng/l. These values are in agreement with those reported by other authors (2, 6, 9).

Stability of plasma catecholamines

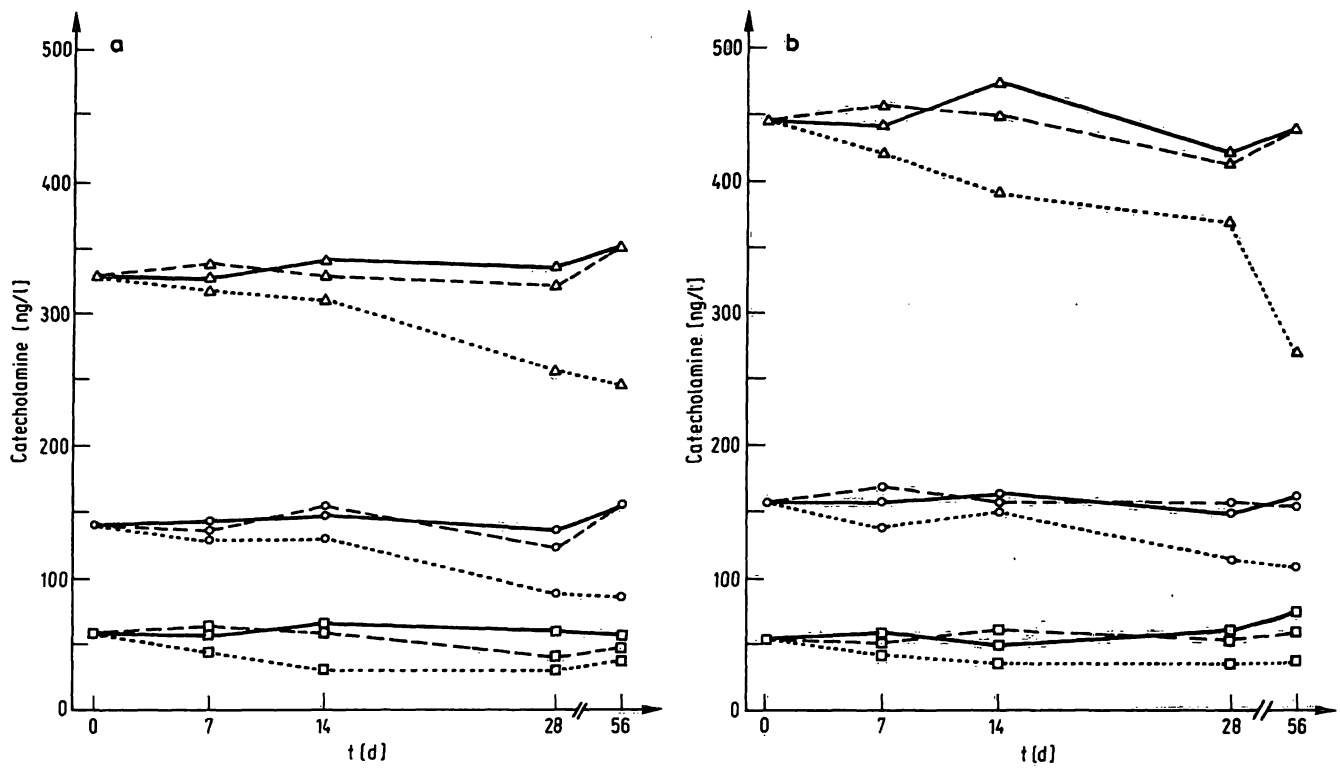
a) Sample collection

Blood was collected with 4 ml Monovettes® (Sarstedt) containing per ml blood: 20 µl Liquemin® and 20 µl of a freshly prepared solution containing 144 mg EGTA and 72 mg reduced glutathione per ml H_2O; the pH of this solution was adjusted to 6.4–7.0 with 6 mol/l NaOH.

The Monovettes® were placed on ice immediately after blood collection and centrifuged within 10 min at 4 °C. Plasma was dispensed in 300 µl-portions in disposable plastic containers (Eppendorf tubes) and stored under different conditions.

b) Sample storage

Plasma samples of two individuals were stored at – 70 °C, – 27 °C and + 4 °C and the plasma of a third subject was stored at room temperature (22 °C).



Figs. 5a and b: Stability of plasma catecholamines of two volunteers in dependence on duration and temperature of storage. (o = epinephrine; Δ = norepinephrine; □ = dopamine; —— stored at – 70 °C – – – stored at – 27 °C ····· stored at + 4 °C).

c) Catecholamine determination

Plasma samples were thawed at room temperature and centrifuged with an Eppendorf portable centrifuge for 1 min. All measurements were carried out in duplicate. The calculation was based on internal standards and $NaIO_4$-oxidized plasma was used for the blanks.

Figures 5 a and 5 b demonstrate that plasma catecholamines are remarkably stable when stored at – 70 °C or at – 27 °C. There is no significant decrease in catecholamine concentration over a period of 8 weeks. Higher storage temperatures lead to a gradual decrease in plasma catecholamine concentrations. After two plasma samples had been stored at + 4 °C for 8 weeks, only 60 (74) % of the norepinephrine, 75 (65)% of the epinephrine and 65 (60)% of the dopamine were found, compared to the initial plasma concentrations immediately after blood sampling. Even if stored at room temperature plasma catecholamines only showed a slight decrease. As a result of these investigations it can be concluded that it is possible to store plasma samples at room temperature for some hours and at – 27 °C for some weeks without losses in catecholamine content.

Stability of catecholamines in blood

10 ml blood were taken from three volunteers. The blood samples were mixed with the anticoagulant, divided into five parts and immediately placed on ice. Plasma of the first blood sample was separated by centrifugation directly after mixing. The other samples were centrifuged 15, 30, 45 and 60 min resp. after venepuncture. Determination of epinephrine and norepinephrine revealed that there was no decay of catecholamine content over the period of study.

Our findings on the stability of catecholamines in blood and plasma are contrary to those of other authors (19), who established a quicker decay of plasma catecholamine content, using a fluorometric method. It may be that this difference is caused by the lower specificity of the fluorometric method in comparison to the radioenzymatic assay.

References

1. Engelman, K., Portnoy, B. & Lovenberg, W. (1968), Am. J. Med. Sci. *255*, 259–268.
2. Engelman, K. & Portnoy, B. (1970), Circ. Res. *26*, 53–57.
3. Passon, P. G. & Peuler, J. D. (1973), Anal. Biochem. *51*, 618–631.
4. Coyle, J. T. & Henry, D. P. (1973), J. Neurochem. *21*, 61–67.
5. Henry, D. P., Starman, B. J., Johnson, D. G. & Williams, R. H. (1975), Life Sci. *16*, 375–384.
6. da Prada, M. & Zürcher, G. (1976), Life Sci. *19*, 1161–1174.
7. Weise, V. K. & Kopin, I. J. (1976), Life Sci. *19*, 1673–1686.
8. Cauchy, C., Tassin, J. P., Glowinsky, J. & Cheramy, A. (1976), J. Neurochem. *26*, 471–480.
9. Peuler, J. D. & Johnson, G. A. (1977), Life Sci. *21*, 625–636.
10. Hörtnagl, H., Benedict, C. R. & Grahame-Smith, D. G. (1977), Br. J. Clin. Pharmacol. *4*, 553–558.
11. Johnson, G. A., Gren, J. M. & Rupiecki, Rosemary (1978), Clin. Chem. *24*, 1927–1930.
12. Saller, C. F. & Zigmond, M. J. (1978), Life Sci. *23*, 1117–1130.
13. Evans, M. I., Halter, J. B. & Porte, D. (1978), Clin. Chem. *24*, 567–570.
14. Axelrod, J. & Tomchick, R. (1958), J. Biol. Chem. *223*, 702–705.
15. Palkovits, M., Brownstein, M., Saavedra, J. M. & Axelrod, J. (1974), Brain Res. *77*, 137–149.
16. Klaniecki, T. S., Corder, C. N., McDonald, R. H. & Feldman, J. A. (1978), J. Lab. Clin. Med. *90*, 604–612.
17. Rentzhog, L. (1972), Acta Physiol. Scand. Suppl. *377/1*, 8–29.
18. Kaiser, H. (1965), Z. Analyt. Chem. *209*, 1–18.
19. Carruthers, M., Taggart, P., Conway, N., Bates, D. & Somerville, W. (1970), Lancet II, 62–67.

Prof. Dr. Dr. H. Wisser
Department of Clinical Chemistry
Robert-Bosch-Krankenhaus
Auerbachstr. 110
D-7000 Stuttgart 50